PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re:	Patent Application of David Norman Leach *et al.*	: Group Art Unit: **1619**
Appln. No.:	10/526,692	: Examiner: **Tigabu Kassa**
Filed:	March 3, 2005	:
For:	EREMOPHILONE AND EREMOPHILONE DERIVATIVES FOR PEST CONTROL	: Attorney Docket No. **DAVI124.001APC**

DECLARATION OF ROBERT SPOONER-HART PURSUANT TO 37 C.F.R. § 1.132

I, Robert Spooner-Hart, of 490 East Kurrajong Road, Kurrajong, New South Wales 2758, Australia, hereby declare as follows:

1. I am the same Robert Spooner-Hart who is a co-inventor of the invention described and claimed in the patent application referenced above.

2. I am an Associate Professor and Leader of the Sustainable Plant Production Systems group at the University of Western Sydney, Sydney, Australia and a Visiting Professor at the Centre of Phytochemistry and Pharmacology at South Cross University, Lismore, New South Wales, Australia.

3. I have read the Office Action dated 22 April 2009 and note that the Examiner appears to consider any compound that kills pests (pesticidal) or repels pests to have antifeedant activity. However, in relation to control of pests, the terms "pesticidal", "pest repellant" and "antifeedant" have different meanings.

4. "Antifeedant" generally means a change in the feeding behaviour of the pest such that they do not feed on a normal food source. The pest may be in contact with a treated surface or a feed source but not feed on it.

5. "Pesticidal" generally means that upon contact or ingestion of a substance death of a pest occurs.

6. "Repellant" generally means that the compound causes the pest to avoid a surface.

7. While there may be some overlap between meanings in that if a pest is killed it will not eat the food source or if the pest avoids a surface it will not use it as a food source, "antifeedant" also encompasses a pest coming into contact with a surface thereby not being repelled and not being killed by the pest-controlling compound but also **not** feeding on the food source. It is also not necessary that the pest starves to death as other food sources may be available.

8. The Examiner has referred to the paper by Gonzalez-Coloma et al. (1995) as evidence that sesquiterpenes have antifeedant activity. However, these authors refer to two uncommon, sesquiterpene compounds, 2,10-bisabolboladien-1-one and 11β-acetoxy-5-angeloxy-silphinene-3-one. In addition, there is only one target insect species, namely the Colorado beetle, *Leptinotarsa decemlineata* Say. This species has limited oligophagy, being restricted to only certain species in one plant family, the Solanaceae. It is, therefore, not surprising that these specific sequiterpenes demonstrated antifeedant activity (as probably would numerous non-sesquiterpene compounds).

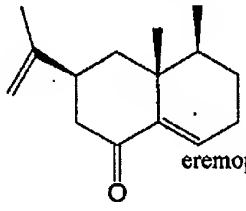
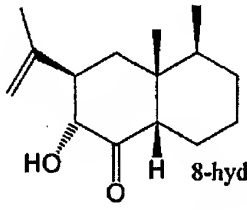
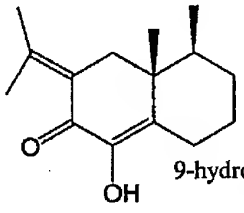
9. A more comprehensive view of the unpredictability of this antifeedant activity is presented in Gonzalez-Coloma et al. (2002), attached as Exhibit RSH-1, in which the activity of ten silphinene sequiterpenes and the sesquiterpene alcohol, farnesol, were assessed against a wider range of insect species. The data presented in this paper assert that even minor modifications to the structure of the silphenene sesquiterpenes significantly alter their antifeedant activity. For example, for *L. decemlineata* and some species of aphid, there were differences in the effective antifeedant dose between the sesquiterpenes of > 275 times and > 5 times, respectively. In addition, the activity of the different sesquiterpenes varied

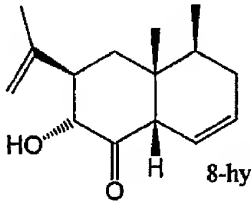
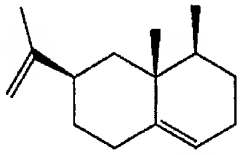
greatly. For example, for 11β-Acetoxy-5ά-isobutyryloxysilphenene-3-one, there was a difference in the effective antifeedant dose between the target insect species tested of > 1000 times. It is also clear that compound 2 did not cause mortality and was therefore not pesticidal, however, had significant antifeedant activity (Gonzalez-Coloma *et al.*, 2002, Table 1 and Table 3, and page 126, First and second full paragraphs).

10. It is also not the case that all sesquiterpenes have pest controlling activity. As can be seen from Gonzalez-Coloma *et al.*, 2002, small changes in structure may significantly affect biological activity.

11. In Table 1 below, I present data relating to the termiticidal activity of 9-carbonyl-eremophilone compounds that supports the unpredictability of bioactivity of sesquiterpene compounds and the importance of the 9-oxo group or a tautomer thereof in the compounds used in the methods of the present application.

Table 1. Sesquiterpene components of *E. mitchellii* and their activity against termites

Compound	Structural formula	LD_{50} (24 hours)
EM-1	 eremophilone	0.16
EM-2	 8-hydroxy-1(10)-dihydroeremophilone	0.68
EM-3	 9-hydroxy-7(11),9-eremophiladien-8-one	0.45

EM-5	 8-hydroxyeremophila-1,11-dienone	0.21
Eremophilene		No mortality
Whole oil distillate	-	0.17

12. The data for the whole oil distillate and EM-1 to EM-3 and EM-5 are derived from Table 4 in Example 3 of the specification. The data for eremophilene is derived from Fraction EM-F1 in Table 3 of Example 2. Fraction EM-F1 is a hydrocarbon fraction having compounds with no oxygenation. The major component of Fraction EM-F1 is eremophilene.

13. As can be seen from these results, eremophilene, a compound with no 9-oxo group and no oxygenation in the 9-position has very little bioactivity. In contrast, EM-1 to EM-3 and EM-5, four compounds of the claimed invention having a 9-oxo group or oxygenation at the 9-position derived from a 9-oxo group (a tautomer), have good bioactivity.

14. In my opinion, these published and presented data demonstrate that someone skilled in control of pests would not be able to conclude, nor predict, antifeedant activity or other kinds of bioactivity of a diverse group of compounds such as the sesquiterpenes, or even eremophilone compounds having 9-oxo substitution or a tautomer thereof, based on the activity of other sesquiterpenes.

15. I declare further that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title

18 of the United States Code and that such wilful false statements may jeopardize the validity of the application or any patent issuing therefrom.

Date: 24 September, 2009

Signature of ROBERT SPOONER-HART

Exhibit RSH-1

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SILPHINENE SESQUITERPENES AS MODEL INSECT ANTIFEEDANTS

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Abstract—Silphinene sesquiterpenes are established chrysomelid antifeedants. In this work, nine silphinene analogs, 11β-acetoxy-5α-angeloyloxysilphinen-3-one (1), 11β-acetoxy-5α-tigloyloxysilphinen-3-one (2), 11β-acetoxy-5α-isobutyryloxysilphinen-3-one (3), 11β-hydroxy-5α-angeloyloxysilphinen-3-one (4), 11β,5α-dihydroxysilphinen-3-one (5), 11β,5α-diacetoxysilphinen-3-one (6), 5α,11β-diisobutyryloxysilphinen-3-one (7), silphinen-3,5,11-trione (8), and *O*-methyl-5-epicantabrenolic acid methyl ester (10), and a presilphiperfolane sesquiterpene (9) were tested against several divergent insect species, including the lepidopteran *Spodoptera littoralis*, the chrysomelid *Leptinotarsa decemlineata*, and five aphid species, and their antifeedant effects were compared with those of picrotoxinin, a GABA-antagonist, and thymol, an allosteric modulator for insect GABA receptors. All insects tested responded to at least one silphinene analog and/or GABA antagonist. Compound 3 and thymol were effective antifeedants against all species tested except *S. littoralis*, with varying potencies according to their feeding ecologies. The toxicity of these compounds was species-dependent and did not correlate with their antifeedant effect.

Key Words—Silphinene sesquiterpenes, picrotoxinin, thymol, GABA inhibitor, antifeedants, Colorado potato beetle, aphids.

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INTRODUCTION

The role of insect taste on host-plant acceptance is basic to the understanding of insect–plant interactions (Bernays and Chapman, 1994). In most cases, antifeedant compounds play an important role in host-plant selection by phytophagous insects (Bernays and Chapman, 1977, 1994, 2000; Mitchell, 1994). The molecular basis of insect taste, however, remains unknown.

Gustatory studies carried out on western corn rootworm (*Diabrotica virgifera virgifera* LeConte, WCR) have shown a direct correlation between antifeedant potency and known antagonistic action at GABA (γ-aminobutyric acid)/glycine amino-acid neuroreceptors (Mullin et al., 1992, 1994; Chyb et al., 1995; Eichenseer and Mullin, 1997). These antagonists include isoquinoline and indole alkaloids and sesquiterpenes (picrotoxinin).

Tricyclic silphinene sesquiterpenes such as compounds **1**, **4**, and **5** (Figure 1) are strong antifeedants to the Colorado potato beetle CPB; (*Leptinotarsa decemlineata* Say) (González-Coloma et al., 1995, 1997). A comparative study between the western corn rootworm and CPB included these silphinenes among 15 compounds, such as alkaloids, terpenoids, and phenolics. The silphinene analogs and all the established GABA- and glycinergic compounds tested acted as antifeedants to both insect species, suggesting a shared molecular mechanism for antifeedant taste chemoreception in divergent Chrysomelidae species (Mullin et al., 1997).

Additionally, sesquiterpenes with strong antifeedant effects on *L. decemlineata*, such as drimanes and bisabolanes, are also active feeding inhibitors to the aphid *Myzus persicae* Sulz (Griffith and Pickett, 1980; Caprioli et al., 1987; Gutiérrez et al., 1997), indicating that CPB sesquiterpene antifeedants are good aphid-antifeedant candidates.

Here we studied the antifeedant effects of 10 silphinene sesquiterpenes against several divergent insect species, including the lepidopteran *Spodoptera littoralis* Bois, the chrysomelid *L. decemlineata*, and five aphid species with diverse host adaptations. The molecules tested are shown in Figure 1 and included 11β-acetoxy-5a-angeloyloxysilphinen-3-one (**1**), 11β-acetoxy-5α-tigloyloxysilphinen-3-one (**2**), 11β-acetoxy-5α-isobutyryloxysilphinen-3-one (**3**), 11β-hydroxy-5α-angeloyloxysilphinen-3-one (**4**), 11β,5α-dihydroxysilphinen-3-one (**5**), 11β, 5α-diacetoxysilphinen-3-one (**6**), 5α,11β-diisobutyryloxysilphinen-3-one (**7**), silphinen-3,5,11-trione (**8**), the presilphiperfolane **9**, and silphinene *O*-methyl-5-epicantabrenolic acid methyl ester (**10**). Compounds **1–4**, **9**, and **10** are natural products, and their biological activities have been compared with those of the semisynthetic ones (**5–8**) to establish their structure–activity relationships. Their antifeedant effects have been compared with those of picrotoxinin, an established GABA-gated chloride channel antagonist (Klunk et al., 1983; Ozoe and Matsumura, 1986) and thymol, an allosteric modulator for insect GABA receptors

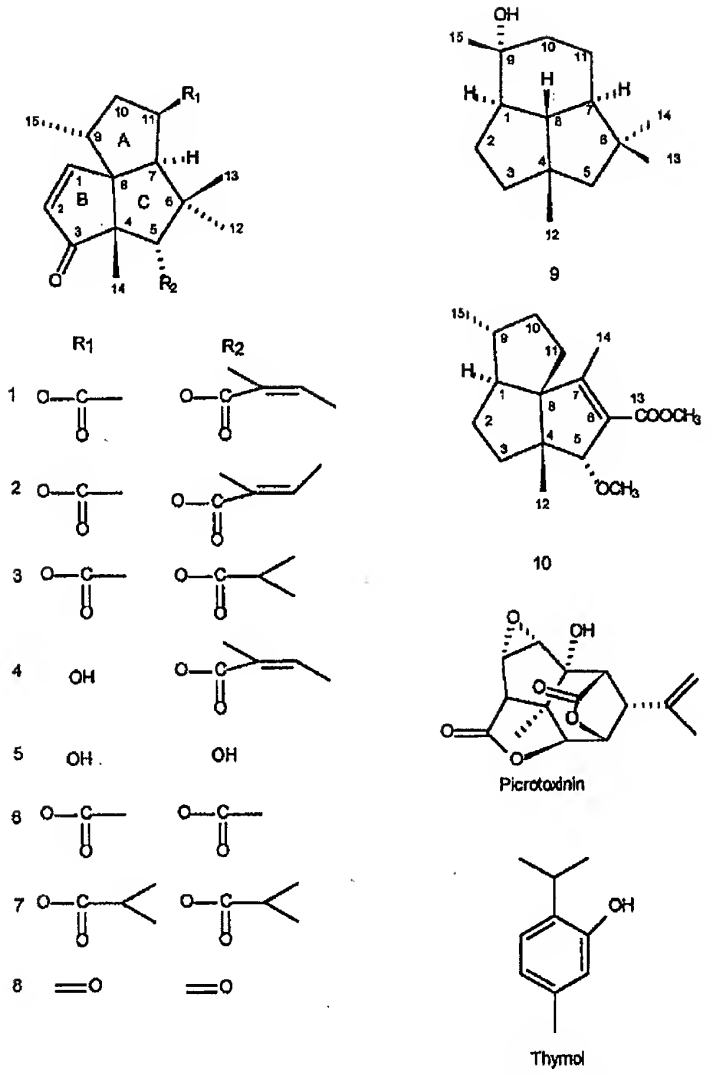

FIG. 1. Molecular structures of compounds **1–10**, picrotoxinin, and thymol.

(Priestley et al., 1999). The sesquiterpene farnesol was included as a positive control for aphid antifeedants (Gutiérrez et al., 1997).

METHODS AND MATERIALS

General Experimental Procedures

NMR spectra were measured on a Bruker AMX2 500-MHz spectrometer with pulsed-field gradient, using the solvent as internal standard ($CDCl_3$, at δ_H 7.26 and δ_C 77.0). Exact mass measurements and electronic impact–mass spectroscopy (EI-MS) results were recorded on an Autospect instrument at 70 eV. HPLCs were carried out on a Gilson-302, equipped with a 115 model UV detector and a Waters Prep-LC 4000 equipped with a diode-array detector. Silica gel from Merck (15111, 7741, 5550) and Aldrich (9,944-3) were used for column chromatography and TLC. Sesquiterpenes were visualized on TLC with a 25% H_2SO_4 solution.

Insects

S. littoralis, *L. decemlineata*, and the aphid colonies (*M. persicae*, *Diuraphis noxia*, *Rhopalosiphum padi*, *Metopolophium dirhodum*, and *Sitobion avenae*) were reared on artificial diet (Poitout and Bues, 1974) and their respective host plants (*Solanum tuberosum*, *Capsicum annuum*, and *Hordeum vulgare*) and maintained at 22 ± 1°C, >70% relative humidity, and photoperiod of 16L:8D in a growth chamber.

Choice Feeding Assays

These experiments were conducted with *S. littoralis* L6 larvae, adult *L. decemlineata*, and apterous aphid adults. For *S. littoralis* and *L. decemlineata*, each treatment consisted of 5–10 Petri dishes with 2 or 4 insects each as described in González-Coloma et al. (1997). The uneaten leaf disk surfaces were measured according to Escoubas et al. (1993) with a computer interfaced scanner. Percent feeding reduction (%FR) was determined for each Petri dish by the equation %FR = [1 − (treatment consumption/control consumption)] × 100 (Bentley et al., 1984). For the aphids, each treatment consisted of 20 boxes with 10 insects each as described in Gutiérrez et al. (1997). A settling inhibition index (%SI) was calculated for each compound [$\%SI = 1 - (\%T/\%C) \times 100$, where $\%T$ is percent aphids on treated surface, $\%C$ is percent aphids on control surface] (Gutiérrez et al., 1997). Compounds with FR or SI > 60% at an initial dose of 50 $\mu g/cm^2$ were tested in a dose–response experiment to calculate their relative potencies (EC_{50} values, the effective dose for 50% feeding/settling reduction), which was determined from linear regression analysis (%FR or %SI on log dose).

Oral Cannulation

This experiment was performed with preweighed newly emerged *S. littoralis* L6 larvae under the same environmental conditions as above. Each experiment consisted of 20 larvae orally dosed with 20 μg of the test compound in 2 μl DMSO (treatment) or solvent alone (control) as described in González-Coloma et al. (1998). At the end of the experiments (72 hr), larval consumption and growth were calculated on a dry weight basis (see González-Coloma et al., 1998 for details). An analysis of covariance (ANCOVA) was performed on biomass gains and food consumption with initial biomass as covariate (Raubenheimer and Simpson, 1992; Horton and Redak, 1993).

Hemolymph Injection

DMSO solutions of the test compounds (10 μg each per insect) were injected through the metepimeron suture of the thorax of 20 adult *L. decemlineata* beetles as described in González-Coloma et al. (1998). Toxicity symptoms and mortality were recorded up to three days after injection by maintenance of beetles on their respective potato leaf foods. Percent mortality was analyzed with contingency tables and corrected according to Abbott (1925).

Isolation of Compounds

Compounds **1**–**4** were isolated from *Senecio palmensis* Chr. Sm. (Asteraceae) (collected in Boca Tauce, Tenerife, July 1996, voucher number ORT 36393, Jardín Botánico de La Orotava, Tenerife). Dried aerial parts of *S. palmensis* (2.8 kg) were exhaustively extracted with EtOH. The ethanolic extract (1000 g, 35.7% yield of dry plant weight) was chromatographed on a silica gel (Si gel) vacuum liquid chromatography (VLC) column using an *n*-hexane–EtOAc–MeOH gradient (*n*-hexane–EtOAc, 100:0–50:50) to give fractions A–G.

Fraction D, 14.5 g (1.45% yield, *n*-hexane–EtOAc, 95:5) was subsequently purified by Si gel-VLC, Sephadex LH-20, Si gel-column chromatography (CC), and semiprep normal-phase HPLC chromatography using a 250- × 20-mm silica column (Inertsil Prep-Sil, 10 μm particle size) at a flow rate of 10 ml/min and an isocratic mixture of Cl_2CH_2–$CNCH_3$ (92:8) to obtain 2.0 g of a mixture of **1** and a minor compound (0.21% yield). Peaks were detected at 254 nm. One hundred milligrams of this mixture were further purified by semiprep reversed-phase HPLC chromatography using a 250- × 20-mm silica column (Kromasil C18, 5-μm particle size) at a flow rate of 20 ml/min and a $CNCH_3$–H_2O gradient (65:35–100:0) to obtain 56 mg of pure **1** (0.11% yield) and 4 mg of pure **2** (5.3×10^{-3}% yield). Peaks were detected at 235 nm.

Fraction E, 70 mg (1.8 g, 0.06% yield, *n*-hexane–EtOAc, 90:10) was purified by semiprep normal-phase HPLC chromatography using a 250- × 8-mm silica

column (Nucleosil, 10-μm particle size) at a flow rate of 1.5 ml/min and a *n*-hexane–EtOAc gradient (92:8–90:10) to obtain 10.9 mg of a mixture (0.01% yield). This mixture was further purified by a gradient (Cl_2CH_2–$CNCH_3$, 100:0–96:4) and a subsequent isocratic (Cl_2CH_2–$CNCH_3$, 97:3) HPLC elution to yield compound **3** (6.5 mg, 5.9×10^{-3}% yield). Peaks were detected at 254 nm.

Fraction F, 1.8 g (6.3×10^{-2}% yield, *n*-hexane–EtOAc, 90:10) were subsequently purified by VLC and Si gel CC and eluted with *n*-hexane–EtOAc mixtures. The eluate obtained with *n*-hexane–EtOAc 85:15 afforded 60 mg of **4** (2.1×10^{-3}% yield).

Compound **5** was generated from **1** by chemical hydrolysis (González-Coloma et al., 1997). Compound **5** (100 mg) was acetylated with Ac_2O-pyridine at room temperature for 24 hr to give a residue of 142 mg. This residue was chromatographed over silica-gel to yield compound **6** (128.2 mg, 95.95%). A mixture of **5** (10 mg), pyridine (0.1 ml), and isobutyric anhydride (0.4 ml) was kept at room temperature for 72 hr to afford the reaction product, compound **7** (11.3 mg, 72.1%), after purification over a silica-gel column. A mixture of **5** (10.6 mg), pyridine (0.2 ml), and Cornforth's reagent (0.5 ml) was kept at room temperature for five days. The excess of reagent was decomposed with EtOH, the solvent removed, and the residue chromatographed over silica-gel column eluting with hexane–EtOAc (60:40), to give compound **8** (6.0 mg, 58%).

Compounds **9** and **10** were previously isolated from *Artemisia chamaemelifolia* Vill. (Asteraceae) (Marco et al., 1996) and provided by Prof. J. A. Marco (U. de Valencia, Spain).

Identification of Compounds

Compounds **1**, **4**, and **5** were identified as *11β-acetoxy-5α-angeloyloxysilphinen-3-one, 11β-hydroxy-5α-angeloyloxysilphinen-3-one, and 11β,5α-dihydroxysilphinen-3-one* by comparing their spectra with previously published data (Jakupovic and Abraham, 1985; González-Coloma et al., 1995, 1997).

11β-Acetoxy-5α-tigloyloxysilphinen-3-one (2). 1.8 mg. oil; EI-MS (70 eV, *m/z*, rel. int., %); 374 (M^+) (1) HRMS *m/z* (rel. int., %): $[M]^+$ 374.2106 (1), for $C_{22}H_{30}O_5$ (calcd. 374.2093). ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 0.91 (3H, s, H-12), 0.94 (3H, d, $J = 6.2$ Hz, H-15), 0.95 (3H, s, H-13), 1.23 (3H, s, H-14), 1.59, (H, ddd, $J = 12.5, 6.0, 3.0$ Hz, H-10α), 1.83 (3H, d, $J = 7.0$ Hz, H-4'), 1.89 (3H, br s, H-5'), 2.10 (H, dd, $J = 13.0, 6.0$ Hz, H-10β), 2.18 (H, s, OCC<u>H</u>$_3$), 2.24 (H, d, $J = 4.4$ Hz, H-7), 2.63 (H, m, H-9β), 5,27 (H, br t, $J = 3.3$ Hz, H-11α), 5.44 (H, s, H-5β), 6.15 (H, d, $J = 6.2$ Hz, H-2), 7.60 (H, d, $J = 6.2$ Hz, H-1), 7.0 (H, dq, $J = 7.0$ Hz, H-3'). ^{13}C NMR ($CDCl_3$, 50 MHz): δ_C 211.1 (s, C-3), 173.9 (s, C-1''), 168.0 (s, C-1'), 167.8 (d, C-1), 138.0 (d, C-3'), 131.9 (d, C-2), 86.3 (d, C-5), 76.1 (d, C-11), 63.7 (s, C-8), 61.5 (d, C-7), 57.0 (s, C-4), 42.6 (s, C-6),

41.7 (t, C-10), 35.0 (d, C-9), 25.0 (q, C-12), 23.7 (q, C-13), 21.7 (q, CO<u>C</u>H$_3$), 19.6 (q, C-14), 15.9 (q, C-15), 14.5 (q, C-4′) 12.1 (q, C-5′).

11β-Acetoxy-5α-isobutyryloxysilphinen-3-one (3). Oil; EI-MS (70 eV, *m/z*, rel. int.,%); 362 ((3), 320 (8), 292 (42), 291 (100), 250 (22), 231 (54), 204 (46), 161 (52), 123 (49) and 83 (82); HRMS *m/z* (rel. int.,%): [M]$^+$ 362.1918 (3), for $C_{21}H_{30}O_5$ (calcd. 362.2093). ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H, 7.55 (1H, d, J = 5.8 Hz, H-1), 6.10 (1H, d, J = 5.5 Hz, H-2), 5.31 (1H, s, H-5β), 5.21 (1H, br t, J = 3.2 Hz, H-11α), 2.64 (1H, qq, J = 7.0 Hz, H-2′), 2.59 (1H, m, H-9β), 2.19 (1H, d, J = 4.4 Hz, H-7α), 2.12 (3H, s, AcO), 2.06 (1H, br dd, J = 13.6, 6.3 Hz, H-10β), 1.56 (1H, ddd, J = 12.0, 3.0 Hz, H-10α), 1.24 (3H, d, J = 7.0 Hz, H-2′), 1.20 (3H, d, J = 7.0 Hz, H-3′), 1.18 (3H, s, H-14), 0.92 (3H, d, J = 7.5 Hz, H-15), 0.89 (3H, s, H-13), 0.86 (3H, s, H-12). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 218.3 (s, C-3), 169.9 (s, <u>C</u>OCH$_3$), 167.7 (d, C-1), 165.6 (C-1′), 131.7 (d, C-2), 86.0 (d, C-5), 76.1 (d, C-11), 63, 8 (s, C-8), 61.5 (d, C-7), 56.9 (s, C-4), 42.4 (s, C-6), 41.7(t, C-10), 35.0 (d, C-9), 34.2 (d, C-2′), 25.0 (q, C-12), 23.7 (q, C-13), 21.7 (q, CO<u>C</u>H$_3$), 19.8 (q, C-4), 19.1 (q, C-3′), 18.9 (q, C-4′), 15.9 (q, C-15).

11β,5α-Diacetoxysilphinen-3-one (6). Oil; EI-MS (70 eV, *m/z*, rel. int.); 334 (M$^+$) (18), 292 (77), 250 (32), 232 (24), 221 (31), 204 (52), 161 (61), 122 (100), 83 (33), and 82 (61). HRMS *m/z* (rel. int.,%): [M]$^+$ 334.1781 (18), for $C_{19}H_{26}O_5$ (calcd. 334.1780). ^{1}H NMR ($CDCl_3$, 200 MHz): δ_H 7.60 (1H, d, J = 5.6 Hz, H-1), 6.10 (1H, d, J = 5.6 Hz, H-2), 5.26 (1H, m, H-11α), 5.13 (1H, s, H-5β), 2.60 (1H, m, H-9β), 2.18 (1H, d, J = 5.7 Hz, H-7α), 2.15 and 2.12 (3H each, s, 2 × OAc), 2.00 (1H, d, J = 6.0 Hz, H-10β), 1.69 (1H, ddd, J = 12.1, 3.5 Hz, H-10α), 1.08 (3H, s, H-14), 0.96 (3H, s, H-13), 0.94 (3H, d, J = 6.8 Hz, H-15), 0.91 (3H, s, H-12). ^{1}H NMR ($CDCl_3$, 500 MHz): δ_C 212.9 (s, C-3), 170.1 (s, C=O), 169.8 (s, C=O), 168.7 (d, C-1), 129.4 (d, C-2), 85.8 (d, C-5), 75.0 (d, C-11), 67.3 (s, C-8), 62.5 (d, C-7), 59.9 (s, C-4), 44.9 (s, C-6), 42.8 (t, C-10), 35.4 (d, C-9), 31.3 (q, C-13), 21.7 (q, CO<u>C</u>H$_3$), 20.8 (q, CO<u>C</u>H$_3$), 20.5 (q, C-14), 15.4 (q, C-15), 15.2 (q, C-12).

5α,11β-Diisobutyryloxysilphinen-3-one (7). Oil; EI-MS (70 eV, *m/z*, rel. int., %); 390 (3), 320 (22), 319 (41), 250 (13), 249 (46), 232 (13), 231 (21), 204 (39), 162 (18), 161 (32), 123 (26), 122 (46), 91 (15), 82 (42), and 71 (100); HRMS *m/z* (rel. int.,%): [M]$^+$ 390.2411 (1), for $C_{23}H_{34}O_5$ (calcd. 390.2406). ^{1}H NMR ($CDCl_3$, 500 MHz) δ_H, 7.56 (1H, d, J = 6.1 Hz, H-1), 6.04 (1H, d, J = 6.1 Hz, H-2), 5.29 (1H, br t, J = 3.0 Hz, H-11α), 5.05 (1H, s, H-5β), 2.62 [2H, m, 2 × OCC<u>H</u> (CH$_3$)2] , 2.60 (1H, m, H-9β), 2.17 (1H, d, J = 6.1 Hz, H-7α), 2.02 (1H, dddd, J = 13.9, 6.6, 2.7 Hz, H-10β), 1.75 (1H, ddd, J = 11.6, 5.0 Hz, H-10α), 1.23 and 1.18 [3H each, J = 7.2 Hz, CH(CH$_3$)$_2$] 1.21, and 1.15 [3H each, d, J = 6.2 Hz, CH(C<u>H</u>$_3$)$_2$], 1.08 (3H, s, H-14), 0.98 (3H, s, H-13), 0.90 (3H, s, H-12), 0.89 (3H, d, J = 6.7 Hz, H-15) ^{13}C NMR ($CDCl_3$, 50 MHz): δ_C 207.9 (s, C-3), 176.7 (s, O<u>C</u>OR), 176.1 (s, O<u>C</u>OR), 168.1 (d, C-1), 129.0 (d, C-2), 84.3

(d, C-5), 74.9 (d, C-11), 67.4 (s, C-8), 61.9 (d, C-7), 59.4 (s, C-4), 45.9 (s, C-6), 42.6 (d, C-9), 35.2 and 34.2 (d, OCOCH-), 31.3 (t, C-10), 20.2 (q, H-13), 19.5, 19.6 and 19.0 (4q, $(CH_3)_2$), 18.4 (q, H-12), 16.0 (q, H-14) and 15.0 (q, H-15).

Silphinen-3,5,11-trione (8.) Oil; EI-MS (70 eV, *m/z*, rel. int., %); 246 (M^+), (20), 218 (17), 203 (5),176 (6), 161 (10), 123 (11), 122 (26), 121 (62), 98 (11), 91 (18), and (100). HRMS *m/z* (rel. int.,%): $[M]^+$ 246.1248 (21), for $C_{15}H_{18}O_3$ (calcd. 246.1255). ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 1.05 (3H, s, H-12), 1.08 (3H, s, H-13), 1.14 (3H, d, $J = 7$ Hz, H-15), 1.22 (3H, s, H-14), 2.29 (1H, br dd $J = 15.9$, 3.3 Hz, H-10α), 2.47 (1H, s, H-7α), 2.49 (1H, br dd $J = 15.5$, 7.8 Hz, H-10β), 6.16 (1H, d, $J = 6.1$ Hz, H-2), 7.87 (1H, d, $J = 6.1$ Hz, H-1). ^{13}C NMR ($CDCl_3$, 50 MHz): δ_C 214.0 (s, C-5 and C-11), 212.6 (s, C-3), 166.7 (d, C-1), 130.3 (s, C-2), 65.5 (s, C-8), 61.6 (s, C-4), 61.0 (d, C-7), 51.6 (s, C-6), 48.2 (d, C-9), 33.5 (t, C-10), 28.0 (q, C-13), 22.2 (q, C-14), 18.0 (q, C-15), 17.9 (q, C-12).

RESULTS AND DISCUSSION

Compounds **1–4** were previously isolated from *Cineraria geifolia*, and Compound (**1**) from *S. palmensis* (Jakupovic and Abraham, 1985; González-Coloma et al, 1995). Compound **4** has also been obtained as the partial hydrolysis product of **1** (González-Coloma et al., 1997), and is being described here as a natural metabolite of *S. palmensis* for the first time. Silphinene derivatives **5–8** were synthesized to carry out structure–activity studies.

The antifeedant effects of compounds **1–10**, picrotoxinin, and thymol were species-dependent (Table 1). The polyphagous *S. littoralis* was moderately sensitive to silphinenes **6** and **8** and insensitive to the GABA antagonists. *L. decemlineata*, a specialist of some Solanaceae species (Mitchell and Harrison, 1985; Hsiao, 1986; Sinden et al., 1988), was strongly sensitive to all the sesquiterpenes and GABA antagonists. *M. persicae* (more than 40 host-plant families) and *R. padi* (the most polyphagous among the cereal aphids) (Blackman and Eastop, 1985) were sensitive to compound **3** and thymol. *S. avenae* and *M. dirhodum*, both specialists of grasses and cereals as secondary hosts (Blackman and Eastop, 1985), were moderately sensitive to silphinenes (*S. avenae* responded to **3** and **5**; *M. dirhodum* to **3**) or picrotoxinin (*M. dirhodum*), and thymol (both). *D. noxia*, with the most restricted host-range (restricted to wheat and barley) (Blackman and Eastop, 1985), was the most sensitive aphid to silphinene derivatives (**1**, **3**, and **4**), picrotoxinin, and thymol. A similar species-dependent pattern was observed for the aphid antifeedant farnesol. Compound **3** was the only silphinene derivative that maintained its antifeedant activity among all the species tested with varying potencies according to their feeding ecologies. These results are consistent with the model suggesting that differences in taste sensitivity to deterrent compounds could account for the difference in host range (Bernays and Chapman,1994; Bernays et al., 2000). The fact that

TABLE 1. EFFECTIVE ANTIFEEDANT DOSES (EC_{50}) AND 95% CONFIDENCE LIMITS (LOWER, UPPER) OF TEST COMPOUNDS ON *S. Littoralis* L6 LARVAE, ADULT *L. Decemlineata*, AND FIVE SPECIES OF APTEROUS ADULT APHIDS

	Ec_{50} (nmol/cm^2) (95% CL)						
Compound	*S. littoralis*	*L. decemlineata*	*M. persicae*	*R. padi*	*S. avenae*	*M. dirhodum*	*D. noxia*
1	>140	0.8 (0.4,1.2)	>150	>150	>150	>150	31.4 (16.3, 60.4)
2	>200	0.17 (0.04, 0.6)	na[a]	na	na	na	>120
3	~100	0.08 (0.03,0.3)	29.3 (9.7,87.9)	~100	13.7 (6.6, 28.5)	35.8 (12.9, 99.4)	8.0 (4.8, 13.3)
4	>150	21.6 (7.4, 62.6)	>150	>150	>150	>150	38.8 (25.0, 61.1)
5	>200	4.8 (1.5, 15.3)	~100	>200	59.6 (3.6,110.4)	>200	>200
6	38.6 (12.3, 121.8)	1.0 (0.4, 2.5)	>150	>150	>150	>150	~100
7	>140	5.6 (2.3, 11.5)	>150	>150	>150	na	~140
8	42.4 (17.05, 106.0)	>100	na	na	na	na	>200
9	na	19.5 (12.2, 31.2)	na	na	na	na	47.5 (26.7, 84.5)
10	na	23.7 (11.4, 49.6)	na	na	na	na	na
Picrotoxinin	>150	27.5 (9.4, 80.1)	>100	>100	>100	53.1 (31.8, 88.6)	16.2 (6.6, 39.4)
Thymol	>300	3.6 (1.1, 12.9)	30.9 (20.8, 45.9)	23.7 (15.4, 36.6)	34.9 (26.1, 59.9)	32.0 (22.3, 45.8)	6.4 (3.6, 11.1)
Farnesol	>200	107.3 (59.9, 192.6)	67.1(54.5, 82.4)	7.9 (5.2, 11.9)	13.1 (8.1, 21.2)	3.7 (1.6, 8.5)	0.2 (0.02, 1.4)

[a]na, not enough compound available.

the insects were sensitive to either a silphinene analog, thymol, and/or picrotoxinin supports the hypothesis of a shared molecular mechanism for antifeedant taste chemoreception in divergent insect species as proposed by Mullin et al. (1997).

L. decemlineata responded to most of the silphinene analogs, as expected from their structural similarities with **1** and their possible biogenetic relationship (Marco et al., 1996), allowing for the study of their structure–activity relationships (Table 1). When compared to **1**, analogs **2** and **3** were 4 and 10 times more active, respectively, **6** was similarly active; and **7**, **9**, and **10** were 6–20 times less active.

Small structural changes resulted in drastic differences in antifeedant activity, suggesting a high molecular selectivity for silphinene derivatives on CPB taste chemoreception. The increased activity of compounds **2** and **3** versus **1** and **6** demonstrated that a small change at the C-5 substituent (from an angelate in **1** or an acetate in **6**, to a tiglate in **2** or an isobutyrate in **3**) had a great impact on the antifeedant poteney. The reduction in activity found for compounds **4**, **5**, and **7** in contrast to **1–3** and **6** showed the importance of the C-11 acetate. The lack of activity of **8** (with carbonyl groups in C-5 and C-11) in contrast with **5** suggests that hydrogen-bonding at C-5 (with a hydroxyl substituent) may play a role in the antifeedant effect of these molecules. The activity of the related silphinenes **9** and **10** indicated that structurally related tricyclic sesquiterpenes are good CPB antifeedant candidates for future studies.

The GABA modulators thymol and picrotoxinin were also CPB antifeedants, with thymol being seven times more active than picrotoxinin (Table 1). Silphinenes **1–3** and **6** were more active than both antagonists; **1**, **5**, and **7** were as active as thymol; while **4**, **9**, and **10** had similar activity levels to picrotoxinin. Silphinene **1** has 3D structural features similar to those of picrotoxinin (Mullin et al., 1997). Furthermore, **1** and **4** are GABA antagonists at mammalian receptors. (Bloomquist, 2001), **4** being threefold more active than **1**, and **5** essentially inactive. These results paralleled their rootworm antifeedant action and effects on excitation of galeal chemoreceptors (Mullin et al., 1997), supporting the hypothesis of a GABA-mediated taste regulation in chrysomelid insects. However, the CPB antifeedant effects of these compounds did not correlate with their mammalian GABA antagonist effect, probably due to the different pharmacology of mammal and insect GABA receptors (see Bloomquist, 2001)

Table 2 shows the toxic and postingestive effects of the test compounds to *L. decemlineata* and *S. littoralis*, respectively. Beetle mortality significantly increased when injected with compounds **3**, **5**, **6**, **9**, and thymol, with **6** being the most toxic, but their toxicity levels did not correlate with their antifeedant action, as previously described for the toxicity of **1**, **4**, and **5** (Gonzalez-Coloma et al., 1997; Mullin et al., 1997), indicating that these compounds could act on both the peripheral and central nervous system of insects. None of the test compounds had negative effects on *S. littoralis* biomass gain or consumption, probably due to metabolic detoxification and/or species-related target differences.

TABLE 2. TP INJECTED (10 μg/INSECT, 20 INSECTS) AND ORAL (20 μg/INSECT, 20 INSECTS), TEST COMPOUNDS ON ADULT *L. decemlineata* AND *S. littoralis* L6 *Larvae*[a]

Compound	*L. decemlineata*	*S. littoralis*	
	Mortality at 72 hr (%)	ΔB[b] (% control)	I[c] (% control)
1	0	102.31 ± 6.17	108.32 ± 5.77
2	0	84.03 ± 7.28	83.67 ± 40.80
3	40[d]	88.77 ± 4.91	93.56 ± 14.25
4	29	90.70 ± 6.17	95.00 ± 5.77
5	35[d]	99.85 ± 6.34	97.53 ± 5.92
6	63[d]	84.92 ± 3.66	117.84 ± 15.68
7	0	86.15 ± 7.23	95.20 ± 32.10
8	0	85.74 ± 8.75	81.49 ± 50.00
9	47[d]	109.86 ± 5.68	93.35 ± 2.58
10	12	na[e]	na
Picrotoxinin	21	104.75 ± 11.14	110.46 ± 11.14
Thymol	36[d]	89.85 ± 8.19	86.91 ± 36.4
P[f]		>0.05	>0.05

[a]Mortality data were corrected according to Abbott (1925).
[b]ΔB = change in insect body weight (dry weight, mg).
[c]I = mg food consumed (dry weight, mg).
[d]Significantly different from the control, $P < 0.05$, contingency table analysis.
[e]na, not enough compound available.
[f]Treatment P-level, ANCOVA analysis with initial body weight as covariate.

In summary, based on the antifeedant effects of silphinenes, thymol, and picrotoxinin against a wide range of insect species, the structural similarities between silphinens and picrotoxinin and the action of these sesquiterpenes on mammalian GABA neuroreceptors suggest a shared GABA-mediated taste regulation in these insects. Therefore, the study of the molecular mode of action of this type of sesquiterpenes merits further investigation.

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